HCV genotype 4—an emerging threat as a cause of chronic liver disease in Indian (south) patients

Sukanya Raghuraman a, Priya Abraham a,*, Gopalan Sridharan a, Hubert Darius Daniel a, B.S. Ramakrishna b, R.V. Shaji c

a Departments of Clinical Virology, Christian Medical College, Vellore 632004, Tamil Nadu, India
b Departments of Gastrointestinal Sciences, Christian Medical College, Vellore 632004, Tamil Nadu, India
c Departments of Haematology, Christian Medical College, Vellore 632004, Tamil Nadu, India

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Abstract

Background: Hepatitis C virus (HCV) genotyping is relevant for the delivery of effective antiviral therapy. HCV genotypes are geographically restricted with genotype 4, which is resistant to therapy, traditionally considered to be confined to the Middle East and Africa. We report here on the occurrence of HCV genotype 4 in Indian (South) patients.

Objectives: (1) To highlight the occurrence of HCV genotype 4 in the patient population attending a tertiary care hospital in south India. (2) To ascertain the difference in HCV viral loads and alanine aminotransferase (ALT) values between patients infected with HCV genotype 4 and those infected with the other two most commonly detected genotypes in this patient population viz., HCV genotypes 1 and 3. (3) To assess the genetic relatedness of the Indian strains to Genbank sequences, which we report for the first time.

Study design: The study group consisted of 125 HCV infected, untreated patients who had been genotyped using type specific primers. Eight of the nine samples classified as HCV genotype 4 by this technique were subjected to nucleotide sequencing. Viral load estimations were carried out. Information on possible risk factors and ALT values across genotypes. A phylogenetic tree was constructed and the genetic relatedness of the strains was assessed through sequence analysis.

Results: HCV genotype 4 was detected in nine of 125 (7.2%) patients. Eight of the nine were subjected to nucleotide sequencing and all strains were confirmed as HCV genotype 4. Six of the eight strains were closely related, with two strains being phylogenetically diverse. Conclusions: HCV genotype 4 is detected in a significant minority of HCV infected patients in India. This finding should be considered in designing strategies prior to initiation of therapy in Indian patients infected with HCV.

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Keywords: Hepatitis C virus; Genotype 4; India; Phylogenetic analysis

1. Introduction

The heterogeneity of the hepatitis C virus (HCV) genome has warranted the classification of the virus into different genotypes. It is now well established that there are a number of different genotypes of HCV (Simmonds et al., 1993), which may have important implications in the pathogenesis of disease (Pozzato et al., 1994), response to anti-viral therapy (Hopf et al., 1996), diagnosis (Neville et al., 1997), in molecular epidemiology studies and vaccine development. Genotypes of HCV are thought to be geographically restricted with the exception of genotype 1. HCV genotype 1 and its subtypes are distributed throughout the world. Genotype 2 is mainly found in Europe and type 3 in Europe and Asia. Reports of genotype 4 have mainly emanated from Egypt (Ray et al., 2000), Saudi Arabia, (Shobokshi et al., 1998), Italy (Argentini et al., 2000) and France (Morice et al., 2001). Genotype 5a has been isolated from blood donors...
in South Africa and genotype 6 from the South East Asian countries of Hong Kong and Macau (Davidson et al., 1995; Suyenoy et al., 1995; Zein et al., 1996).

The reason for the geographical restriction of HCV genotypes is yet unclear. The preponderance of a genotype in a particular area may be due to the rapid rate of its spread influenced by certain transmission routes (Simmonds, 2000). We had previously reported on the presence of HCV genotype 4 from India (Raghuraman et al., 2003). Here we elaborate on HCV genotype 4, which has consequences for therapy, from an extended study.

In this study, we report on the sizeable number of patients from south India harbouring HCV genotype 4 strains. The detection of genotype 4 in Indian patients with chronic liver disease has negative consequences, as this genotype is now shown to have a poorer response to interferon (IFN) in comparison to genotypes 2 and 3 (el-Zayadi et al., 1996; Zylberberg et al., 2000).

2. Material and methods

2.1. Patients

Subjects included patients seen at the Christian Medical College, Vellore, TamilNadu between the period 1998–2002 in the Outpatient Departments of Nephrology, Hepatology, Haematology and Internal Medicine. HCV RNA testing was undertaken primarily for patients who had earlier tested positive for antibody to HCV (HCV Ab), or as part of the follow up after renal or bone marrow transplantation.

Of the 219 HCV RNA positive plasma samples obtained during this period, only 125 samples (57%) were analysed further. These 125 samples consisted of plasma obtained from interferon naïve patients, from whom sufficient archived plasma aliquots for further testing was available.

Reverse transcriptase polymerase chain reaction (RT-PCR) for HCV RNA was carried out as previously described from our laboratory. (Radhakrishnan et al., 2000). HCV genotyping and viral load estimation were done additionally as part of another study that investigated the prevalence of different HCV genotypes in relation to virus loads in the patient population attending this referral center (Raghuraman et al., 2003).

Risk factors for the study patients were obtained from their hospital records. A general consent is obtained in our hospital for all patients as part of routine patient management.

2.2. HCV RNA genotyping

Samples were genotyped with type-specific primers designed from the core region of the HCV genome. The protocol for genotyping was carried out as earlier described (Ohno et al., 1997). Samples were assigned genotypes based on the final size of the amplified product, as visualized on an ethidium bromide stained agarose gel.

2.3. HCV RNA sequencing

Of the 125 samples that had been genotyped using the technique described above, nine samples (7.2%) were identified as genotype 4.

In the present study, eight of the nine samples identified as HCV genotype 4 by the type specific primer based genotyping assay, were further investigated by nucleotide sequencing. Bidirectional sequencing was carried out with primers specific for the core region of the genome (Ohno et al., 1997). Cycle sequencing reactions were carried out using the ABI Big Dye Terminator Ready Reaction mix (Applied Biosystems, USA). The cycle sequencing products were purified by ethanol precipitation and finally resuspended in 25 µl of Template Suppression Reagent (Applied Biosystems, USA). Sequences were generated on a ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). The ninth HCV strain could not be sequenced due to lack of adequate volume of plasma.

2.4. Nucleotide sequence analysis

Study sequences were aligned using the sequence navigator software. The HCV genotype was identified using the taxonomy report generated for a sequence after subjecting the sequences to a Basic Blast search. The evolutionary relationship between the isolated sequences was investigated by constructing a phylogenetic tree. Representative sequences from different HCV genotypes were retrieved from GenBank databases (http://www.embl-nl.nhm.ou/permissions/Entrez).

The genetic distances and the phylogenetic tree were computed using the MegAlign programme (DNASTAR Inc., Wisconsin, USA).

2.5. HCV RNA quantitation

The viral loads were estimated in International Units per ml (IU/ml) of plasma using the Amplicor HCV version 2.0 (Roche Diagnostics, Germany). Samples that had viral loads greater than the outer limit of quantitation by the assay (i.e., 850,000 IU/ml of plasma), were diluted and retested as per the manufacturer’s recommendation to obtain precise viral titers.

2.6. Statistical analyses

Age, alanine aminotransferase (ALT) values, as obtained from the patients’ hospital records, were compared using ANOVA. Comparison of proportions was carried out using the Chi-square test. Both statistical tests were carried out with the Epi-Info software (version 6.04b). The average viral loads in patients infected with the different HCV genotypes was compared using the Mann–Whitney U or Wilcoxon Rank-Sum test for difference in medians using the NCSS/PASS 2000 (Dawson Edition) software.
3. Results

Eight HCV strains identified as genotype 4 by type specific primers were confirmed by nucleotide sequencing. The demographic profile of the patients infected with HCV genotypes is shown in Table 1.

The two most commonly prevalent genotypes in the Indian population are HCV genotypes 1 and 3 (Raghuraman et al., 2003), and hence the ALT values and viral titers observed with these two genotypes were used for comparison. Though the difference was not significant, the mean ALT observed in HCV genotype 4 infection was higher as compared to the other 2 genotypes (Table 2).

The average viral loads of patients infected with HCV genotype 1 was significantly higher than the average viral loads of patients infected with HCV genotype 3 (P = 0.03) and HCV genotype 4 (P = 0.04). There was no significant difference in viral titers between patients infected with HCV genotype 3 and those infected with HCV genotype 4 (P = 0.67) (Table 2).

The elicited risk factors for patients with HCV genotype 4 included high-risk sexual behaviour (n = 1), a past history of surgery (n = 1) and a history of having received multiple injections (n = 1). For five patients no particular risk factor could be elicited (Table 1).

Molecular characterization was carried out by sequence analysis and construction of a phylogenetic tree (Fig. 1). Six of the eight sequenced strains (G4001, G4003, G4004, G4006, G4007 and G4008) formed a separate cluster on the phylogenetic tree while two of the eight strains (G4002 and G4005) were seen to group separately.

The genetic relatedness of the Indian genotype 4 isolates was evaluated by estimation of intra and inter group genetic distances and is shown in Table 3. Percentage divergence values ranging from 1.3% to 8.5% was observed between the Indian (South) HCV genotype 4 isolates.

4. Discussion

This report indicates that HCV genotype 4 is encountered in a significant minority (7.2%) of the Indian (South) population. Since only interferon naïve patients’ samples with adequate volume were genotyped it is possible that genotype 4 is present in a higher proportion of patients in this region. The average viral load seen in patients with genotype 4 infection was not significantly different from that seen in genotype 3 patients though patients infected with either genotype 3 or genotype 4 strains had significantly lower viral loads than that seen in genotype 1 infected patients. Interestingly, HCV genotype 4 patients had higher average ALT levels than HCV genotype 1 or HCV genotype 3 infected patients. Coupled together these observations lead one to speculate that the associated liver pathology in genotype 4 infected patients may be due to factors other than increased viral replication.

There have been two published reports of HCV genotype 4 infection from India. In the study by Sawant et al. (1999), HCV RNA was detected in 5 of the 99 cirrhotic patients attending an urban hospital in Mumbai. Three of the five virological samples were sequenced, of which one sample was found to have HCV genotype 4. In another study the presence of HCV genotype 4 was seen in approximately 4% of the group (Das et al., 2002). The present study reiterates the presence of HCV genotype 4 among patients in India (South).

The nine patients with HCV genotype 4 infection, were part of a study group comprised of patients drawn from north India (n = 12), east India (n = 73), and south India (n = 40). In this study group, genotype 4 infection was detected exclusively in south Indian patients. Additionally, a recent study which described the phylogenetic analysis of HCV isolates from 149 chronic carriers from northern, southern, western and eastern India, detected genotype 4 only in south Indian patients (Lole et al., 2003). The detection rate of genotype 4 in Indian (South) patients was significantly higher (P < 0.000, chi-square test), as compared to the detection rates of genotype 1 and genotype 3 from Indian (South) patients (Data not shown). In an earlier published study from this centre, two samples from India (East) were identified as belonging to genotype 4 by the type specific primer based
Fig. 1. Phylogenetic tree showing the evolutionary relationship among the HCV genotype 4 isolates from south Indian patients of the present study (in bold type) with respect to other prototype sequences. These sequences represent the core region of the HCV genome. Prototype sequences are indicated by their GenBank accession numbers with the genotype indicated within parentheses.

Table 3
Percentage divergence between the eight genotype 4 strains\(^a\) isolated from India (South) in the present study and in comparison to other GenBank\(^b\) sequences from the HCV core region\(^c\)

<table>
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<tr>
<th>Strain</th>
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<th>G4003</th>
<th>G4004</th>
<th>G4005</th>
<th>G4006</th>
<th>G4007</th>
<th>G4008</th>
<th>AF29298(4a)</th>
<th>L38338(4e)</th>
<th>L38339(4e)</th>
<th>L38332(4f)</th>
<th>Y11604(4a)</th>
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<td>1.3</td>
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<td>6.9</td>
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</table>

\(^a\) Study sequences are G4001–G4008.
\(^b\) GenBank sequences are indicated by their Accession numbers with the genotype identity indicated in parentheses.
\(^c\) The percentage divergence values of the two divergent strains i.e., G4002 and G4005 in comparison to the remaining strains are shown in bold.
these patients. The identification and treatment of patients able on the viral kinetics, response rates and prognosis for group with the genotype 1 infected patient group for an-
fected patients. This has necessitated the clubbing of this 4 may have entered the Indian population as a result of an unapparent transmission from these returning émigrés.

Schroter et al., 2002 reported an increasing frequency of HCV genotype 4a among 747 German nationals. The ob-
ervation that this genotype was seen at a higher frequency in younger individuals (i.e., aged <40 years) and in intra-
venous drug users had led the authors to predict an accel-
erate spread of this subtype in Germany (Schroter et al., 2002). In the present study, the mode of transmission was not clear with five of the eight patients having no known risk factor. In India, as increasing number of patients be-
come infected with this genotype the mode of transmission may become better delineated.

HCV genotype 4 has been associated with a poor response to interferon in contrast to genotypes 2 & 3 (el-Zayadi et al., 2001, 2002). However, there have been relatively few studies that target HCV genotype 4 in-
fected patients. This has necessitated the clubbing of this group with the HCV genotype 1 infected patient group for an-
tiviral regimens. Thus, there is very little information avail-
able on the viral kinetics, response rates and prognosis for these patients. The identification and treatment of patients infected with HCV genotype 4 is necessary as a large cohort study observed that there is an increased risk of liver related deaths and requirement for liver transplantation in patients infected with genotype 4 (Khan et al., 2000).

In conclusion, the presence of sizeable numbers of pa-
tients infected with HCV genotype 4 and the suggested ge-
etic heterogeneity of this genotype in India merits further inves-
igation. Detailed nucleotide sequencing techniques are im-
portant to delineate the subtype(s) of genotype 4 strains responsible for infection in the Indian subcontinent. Subtype specific differences in pathogenicity, if any, may have to be taken into consideration for estimating prognosis and for-
mulating antiviral therapy regimens. Nucleotide sequencing studies will also assist in tracing sources of spread among south Indian patients. Longitudinal antiviral response stud-
ies, incorporating genotype 4 infected patients, are needed for establishing appropriate treatment protocols in the Indian subcontinent.

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